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(54) Title: ARTERY- AND VEIN-SPECIFIC PROTEINS AND USES THEREFOR			
(57) Abstract <p>Arterial and venous endothelial cells are molecularly distinct from the earliest stages of angiogenesis. This distinction is revealed by expression on arterial cells of a transmembrane ligand, called EphrinB2 whose receptor EphB4 is expressed on venous cells. Targeted disruption of the <i>EphrinB2</i> gene prevents the remodeling of veins from a capillary plexus into properly branched structures. Moreover, it also disrupts the remodeling of arteries, suggesting that reciprocal interactions between pre-specified arterial and venous endothelial cells are necessary for angiogenesis. This distinction can be used to advantage in methods to alter angiogenesis, methods to assess the effect of drugs on artery cells and vein cells, and methods to identify and isolate artery cells nad vein cells, for example.</p>			

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## ARTERY- AND VEIN-SPECIFIC PROTEINS AND USES THEREFOR

## RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Application Number 09/085,820 filed on May 28, 1998, which is a continuation-in-part of U.S. Application Number 09/083,546 filed on May 22, 1998 (abandoned). This application also claims the benefit of U.S. Provisional Application Number 60/081,757 filed on April 13, 1998. The teachings of each of these applications are incorporated herein by reference in their entirety.

## BACKGROUND OF THE INVENTION

The process of blood vessel formation is fundamental in both development and disease. The circulatory system is the first organ system to emerge during embryogenesis, and is necessary to nourish the developing fetus. Disorders of the circulatory system, such as coronary artery disease, are a major cause of morbidity and mortality in modern society. Thus, repairing, replacing and promoting the growth of blood vessels is a major target of clinical research and of pharmaceutical development. Conversely, the ingrowth of new capillary networks into developing tumors is essential for the progression of cancer. Thus, the development of drugs that inhibit this process of tumor angiogenesis is an equally important therapeutic goal. Little attention has been paid to the problem of how arteries and veins acquire their distinct identities. Indeed, many people have assumed that the anatomical and functional differences between arteries and veins simply reflect physiological influences, such as blood pressure, oxygenation and shear forces. Additional

knowledge of how arteries and veins acquire their respective identities would be valuable in both research and clinical settings.

#### SUMMARY OF THE INVENTION

5       The present invention relates to a method of distinguishing between arterial cells (including arterial endothelial cells) and venous cells based on the expression of a protein on arterial cells (arterial endothelial cells) and not on venous cells, and to a  
10 wide variety of processes, methods and compositions of matter, including those useful in research and clinical settings, which are based on the difference in expression between the two cells types. As described herein, it has been shown that there is a molecular  
15 distinction between arterial endothelial cells (arteries) and venous endothelial cells (veins) and that arterial endothelial cells and venous endothelial cells bear molecular markers which can be used to identify, separate, target, manipulate or otherwise process each  
20 cell type specifically (separate from the other). As a result, arteries and veins can now be distinguished from one another, and cell types that make up arteries and veins can be assessed for other genetic molecular or functional differences and targeted, manipulated or  
25 otherwise processed individually or separately for research, diagnostic and therapeutic purposes.

      The present invention relates to methods of distinguishing and separating arterial cells from vein cells, and more specifically, distinguishing and  
30 separating arterial endothelial cells from venous endothelial cells based on their respective molecular markers; methods of selectively targeting or delivering drugs or agents to arteries or veins; methods of altering (enhancing or inhibiting, where "inhibiting"  
35 includes partially or completely inhibiting) the function of artery-specific or vein-specific molecular

markers or interaction between them (and, thus, enhancing or inhibiting the effect such functions or interactions have on arterial endothelial cells or venous endothelial cells); and methods of screening for  
5 drugs which act selectively on arterial cells (and more specifically, on arterial endothelial cells) or venous cells (and more specifically, on venous endothelial cells).

The invention also relates to transgenic nonhuman  
10 mammals, such as transgenic mice, in which genes encoding an arterial cell molecular marker or a venous cell molecular marker are altered, either physically or functionally, and their use as "indicator mice" to specifically visualize either arteries or veins, to  
15 assess the function of the molecular marker which has been altered and to identify drugs which affect (enhance or inhibit) their function. It further relates to antibodies which bind an arterial cell-specific marker or a venous cell-specific marker; viral or other vectors  
20 targeted to arteries or veins by virtue of their containing and expressing, respectively, an arterial cell-specific marker or a venous cell-specific marker; cDNAs useful for preparing libraries to be screened for additional artery- or vein- specific genes, and  
25 immortalized cell lines derived from isolated arterial endothelial cells, from venous endothelial cells, or from transgenic animals (e.g., mice) of the present invention.

A molecular marker for an arterial cell or a venous  
30 cell is any gene product (protein or RNA or combination thereof) expressed by one of these cell types and not by the other. Such a marker can be arterial endothelial cell-specific (artery-specific) or venous endothelial cell-specific (vein-specific) products or proteins. In  
35 specific embodiments, these can be referred to, respectively, as arterial endothelial cell-specific (artery-specific) ligands and venous endothelial cell-

specific (vein-specific receptors. Such molecular markers can be expressed on cell types in addition to arterial or venous endothelial cells, but are not expressed on both arterial and venous endothelial cells.

- 5 Molecular markers can include, for example, mRNAs, members of ligand-receptor pairs, and any other proteins such as adhesion proteins, transcription factors or antigens which are not expressed on both cell types. In one embodiment, the molecular marker is a membrane
- 10 receptor which is the receptor for a growth factor which acts on arteries or veins. In another embodiment the molecular marker is a member of an endothelial cell surface ligand-receptor pair which is expressed on arterial or venous endothelial cells, but not on both.
- 15 For example, as described in detail herein, a member of the Ephrin family of ligands and a member of the Eph family of receptors which is its receptor are molecular markers for arterial endothelial cells and venous endothelial cells, respectively and are useful to
- 20 distinguish the two cell types. Any Ephrin family ligand which is expressed on arterial endothelial cells, but not on venous endothelial cells and a venous endothelial cell-specific Eph family receptor which binds the arterial endothelial cell-specific ligand can
- 25 be used to distinguish between arteries and veins.

In one embodiment, the present invention relates to the discovery that arterial endothelial cells express an Ephrin family ligand and venous endothelial cells express an Eph family receptor which is a receptor of

30 the Ephrin family ligand expressed on the arterial endothelial cells; methods of distinguishing or separating arterial cells (arteries) from venous cells (veins); methods of selectively targeting or delivering drugs or agents to arteries or veins; methods of

35 enhancing or inhibiting angiogenesis, including angiogenesis in tumors, such as by altering (increasing, decreasing or prolonging) activity of at least one

member of an Ephrin family ligand-cognate Eph family receptor pair and drugs useful in the methods; and methods of screening for drugs which selectively act on arteries or veins.

- 5        It further relates to transgenic nonhuman mammals, such as transgenic mice, which have altered genes encoding an Ephrin family ligand or altered genes encoding an Eph family receptor, such as EphrinB2 knockout mice which contain a tau-lacZ (tlacZ) insertion
- 10 that marks arteries but not veins or EphB4 knockout mice which contain a reporter construct (e.g., lacZ or alkaline phosphatase gene) in the EphB4 locus; methods of using these mice as "indicator mice" to define and visualize angiogenic processes (e.g., tumor angiogenesis
- 15 and ischemia-associated cardiac neovascularization) or to screen drugs for their angiogenic or anti-angiogenic effects on arteries or veins in vivo; and cells, such as immortalized cells, derived from the transgenic mice. The present invention also relates to antibodies which
- 20 bind an artery-specific Ephrin family receptor (e.g., antibodies which bind EphrinB2); antibodies which bind a venous-specific Eph family receptor (e.g., antibodies which bind EphB4); viral or other vectors which are targeted to arteries or veins for vessel-specific gene
- 25 therapy by virtue of their containing and expressing DNA encoding, respectively, an Ephrin family ligand (e.g., EphrinB2) or an Eph family receptor (e.g., EphB4); cDNAs useful for preparing libraries to be screened for additional artery-specific or vein-specific genes (whose
- 30 gene products, in turn, might be artery-or vein-specific drug targets) and methods of repairing or replacing damaged arteries or veins by transplantation of isolated arterial or venous endothelial cells, immortalized cell lines derived from them, or synthetic vessels configured
- 35 from these cells.

As described herein and as is known to those of skill in the art, Ephrin family ligands are divided into two subclasses (EphrinA and EphrinB) and Eph family receptors are divided into two groups (EphA and EphB).

5 As is also known, within each subclass or group, individual members are designated by an arabic number. The invention is described herein with specific reference to EphrinB2 and EphB4; However, other Ephrin family ligand-Eph family receptor pairs which show  
10 similar artery-and vein-specific expression and their uses are also the subject of this invention. Similar artery- and vein-specific pairs can be identified by methods known to those of skill in the art.

#### BRIEF DESCRIPTION OF THE DRAWINGS

15 Figure 1A is a diagram of the wild type locus of the EphrinB2 gene showing the Exon-1 structure. The filled box represents 5' untranslated region. The hatched box starts at the ATG, and includes the signal sequence. H=HindIII; X=XbaI; N=NcoI; E=EcoRI.

20 Figure 1B is a diagram of the targeting vector used to disrupt the EphrinB2 gene.

Figure 1C is a schematic representation of the mutated EphrinB2 locus.

Figure 2 is a bar graph indicating the binding  
25 activity to GPI-ephrin-B2 of EphB2Fc in the presence of hamster anti-ephrin-B2 hybridoma supernatants.

#### DETAILED DESCRIPTION OF THE INVENTION

As described herein, it has been shown that arteries and veins are genetically distinct from the  
30 earliest stages of embryonic development and that reciprocal interactions between arteries and veins are essential for proper vessel formation. This finding not only changes dramatically our view of the basic ontogenetic anatomy of embryonic vasculature, but also  
35 provides the means to distinguish between arterial



endothelial cells and venous endothelial cells, both physically and functionally. As a result, means of separating the two cell types from one another; of identifying other artery- or vein-specific genes; of  
5 assessing the selective effects of drugs or other agents on arteries or veins and, thus, identifying those which are artery- or vein-specific; and of selectively delivering or targeting substances to either cell type, are now available. In addition, the work described  
10 herein makes it possible to modulate (enhance or inhibit) or control vasculogenesis and angiogenesis and to do so, if desired, in an artery- specific or vein-specific manner.

As described in the examples, a gene which encodes  
15 a cell membrane-associated ligand which is present in the nervous system and the vascular system has been shown, in adult mice, to be expressed by arterial endothelial cells, and not by venous endothelial cells. Further, the gene which encodes the receptor for the  
20 ligands has been shown to be expressed by venous endothelial cells, but not by artery cells. Thus, for the first time, a marker found on arterial endothelial cells (an artery-specific marker) and a venous endothelial cell- (vein-specific) marker are available,  
25 making it possible to distinguish between arteries and veins for a variety of purposes, such as further study and understanding of the mechanisms of blood vessel formation; selective targeting of treatments or therapies to arteries or veins (targeting to arteries  
30 but not veins or vice versa) and selective modulation (enhancement or inhibition) of formation, growth and survival of arteries and/or veins.

In addition, the work presented in the examples demonstrates that reciprocal signaling between arteries  
35 and veins is crucial for vessel morphogenesis (development/ formation of arteries and veins). As described, deletion of the ligand-encoding gene in mice

prevented the proper development of both arterial and venous vessels. Since the ligand is present on arteries (but not veins), the occurrence of the venous defect is evidence that veins require a signal from arteries for vessel morphogenesis. Conversely, since the arteries are also defective in the mutant mice, the ligand must have a function in the arterial cells themselves, in addition to its role in signaling to the veins. In view of the fact the ligand present on arterial endothelial cells is a transmembrane structure, it most likely functions to receive and transduce to arterial cells a reciprocal signal from venous cells.

Specifically, a ligand which is a member of the Ephrin family of Eph family receptor interactive proteins (Eph family of transmembrane ligands) has been shown to be expressed by arterial endothelial cells, but not by venous endothelial cells. Thus, it is now possible to distinguish between or target arteries and veins by relying on the presence or absence of an Ephrin family ligand and its receptor, which is a member of the Eph family of receptor protein-tyrosine kinases. As described herein, arterial endothelial cells have been shown to express EphrinB2 and venous endothelial cells have been shown to express EphB4, which is an EphrinB2 receptor. EphrinB2 is not expressed on venous endothelial cells and EphB4 is not expressed on arterial endothelial cells, providing a means by which the two cell types can be identified or distinguished and, thus, a means by which arterial endothelial cells and venous arterial cells can be, for example, separated from one another, targeted specifically or acted upon in a selective manner (e.g., by a drug or agent which acts upon one cell type to the exclusion of the other). For example, antibodies that bind to EphrinB2 or to its extracellular domain can be fluorescently labeled and allowed to bind to a mixture of cells, which are then

subjected to fluorescent activated cell sorting to select cells of arteries from the mixture.

The work described herein, particularly in the examples, refers to EphrinB2 and EphB4. However, any  
5 ligand-receptor pair from the Ephrin/Eph family, any other ligand-receptor pair or any gene product produced by one cell type and not the other (e.g., an Ephrin ligand is expressed by arterial endothelial cells but not by venous endothelial cells and an Eph receptor is  
10 expressed by venous endothelial cells but not by arterial endothelial cells) can be used to distinguish between or identify and, thus, selectively act upon, arterial endothelial cells and venous arterial cells.

The ephrins (ligands) are of two structural types,  
15 which can be further subdivided on the basis of sequence relationships and, functionally, on the basis of the preferential binding they exhibit for two corresponding receptor subgroups. Structurally, there are two types of ephrins: those which are membrane-anchored by a  
20 glycerophosphatidylinositol (GPI) linkage and those anchored through a transmembrane domain.

Conventionally, the ligands are divided into the Ephrin-A subclass, which are GPI-linked proteins which bind preferentially to EphA receptors, and the ephrinB  
25 subclass, which are transmembrane proteins which generally bind preferentially to EphB receptors.

The Eph family receptors are a family of receptor protein-tyrosine kinases which are related to Eph, a receptor named for its expression in an erythropoietin-  
30 producing human hepatocellular carcinoma cell line. They are divided into two subgroups on the basis of the relatedness of their extracellular domain sequences and their ability to bind preferentially to ephrinA proteins or ephrinB proteins. Receptors which interact  
35 preferentially with ephrinA proteins are EphA receptors and those which interact preferentially with ephrinB proteins are EphB receptors.

- As used herein, the terms Ephrin and Eph are used to refer, respectively, to ligands and receptors. They can be from any of a variety of animals (e.g., mammals/nonmammals, vertebrates/nonvertebrates, including humans). The nomenclature in this area has changed rapidly and the terminology used herein is that proposed as a result of work by the Eph Nomenclature Committee, which can be accessed, along with previously-used names at web site <http://www.eph-nomenclature.com>.
- 5
- 10 For convenience, eph receptors and their respective ligand(s) are given in the Table.

EPH RECEPTORS AND LIGAND SPECIFICITIES	
<u>Eph Receptors</u>	<u>Ephrins</u>
EphA1	Ephrin-A1
EphA2	Ephrin-A3, -A1, A5, -A4
5 EphA3	Ephrin-A5, -A2, A3, -A1
EphA4	Ephrin-A5, -A1, A3, -A2, -B2, -B3
EphA5	Ephrin-A5, -A1, A2, -A3, -A4
EphA6	Ephrin-A2, -A1, A3, -A4, -A5
EphA7	Ephrin-A2, -A3, A1
10 EphA8	Ephrin-A5, -A3, A2
EphB1	Ephrin-B2, -B1, A3
EphB2	Ephrin-B1, -B2, B3
EphB3	Ephrin-B1, -B2, B3
EphB4	Ephrin-B2, -B1
15 EphB5	Unknown
EphB6	Unknown
Ligand specificities are arranged in order of decreasing affinity. Adapted from Pasquale, E.B. (1997) <i>Curr. Opin. Cell Biol.</i> 9(5)608.	

The work described herein has numerous research and clinical applications, which are discussed below.

As used herein, a transgenic mouse is one which has, incorporated into the genome of some or all its nucleated cells, a genetic alteration which has been introduced into the mouse or at least one of its ancestors, by the manipulations of man. A transgenic mouse can result, for example, from the introduction of DNA into a fertilized mouse ovum or from the introduction of DNA into embryonic stem cells.

One embodiment of the present invention is a transgenic mouse, which because of its particular genotype, expresses only in cells of veins or only in cells of arteries a gene whose RNA transcript or polypeptide gene product can be detected, for example, by in situ hybridization of RNA, by fluorescence, by detection of enzymatic activity, or by detection of a gene product by antibody binding and a detection system for the bound antibodies.

A particular embodiment of the present invention is a transgenic mouse of genotype *EphrinB2*<sup>+/-</sup>, wherein the "minus" allele denotes an allele in which a naturally occurring allele has been deleted, modified or replaced with a mutant allele, including a mutant allele which can have an insertion of an indicator gene. Such a "minus" allele can encode an EphrinB2 ligand which has wild type, altered or no ligand function. A mouse of genotype *EphrinB2*<sup>+/*clac2*</sup> has been produced as described in Example 1 and used to demonstrate that arterial endothelial cells and venous endothelial cells differ genetically from early stages of development and that reciprocal interactions, essential for proper capillary bed formation, occur between the two types of vessels. A transgenic mouse of the same phenotype can be produced by other methods known to those of skill in the art.

Such methods are illustrated below using the EphrinB2 gene as an example, but can also be used for any other vein- or artery-specific gene.

For example, it is possible to produce a vector  
5 carrying an insertion, a deletion, or one or more point mutations in the EphrinB2 gene. The EphrinB2 transgene can be introduced into the genome, via a vector carrying a mutagenized EphrinB2 allele, either by introducing the transgene into a fertilized ovum, by the method of  
10 Wagner et al., US 4,873,191 (1989), or by introducing the transgene into embryonic stem (ES) cells (see, for example, Capecchi, M.R., *Science* 244:1288-1292, 1989), or by other methods.

An insertion of DNA used to construct a transgenic  
15 knockout mouse can have within it a gene whose presence can be readily tested, such as *neo*, which confers upon its host cells resistance to G418. It is an advantage of an *EphrinB2*<sup>+/-</sup> indicator mouse (e.g., *EphrinB2*<sup>+/-</sup>*taulacZ* to be able to express, under the control of the  
20 EphrinB2 promoter, an indicator gene, which can be any gene not endogenously expressed by mice. A particularly advantageous indicator gene is one which facilitates the detection of EphrinB2 expression, presumably as it is occurring in the wild type allele, by the production of  
25 a gene product that is detectable, for example, by its own light absorbance properties, its ability to act upon a substrate to yield a colored product, or its ability to bind to an indicator or dye which is itself detectable.

30 Further, alternative methods are available to produce conditional knockouts or tissue specific knockouts of a gene expressed specifically in veins or in arteries (i.e., a vein-specific or artery-specific gene), for example by a site-specific recombinase such

as Cre (acting at *loxP* site) or FLP1 (acting at *FRT* site) of yeast.

The bacteriophage P1 Cre-*loxP* recombination system is capable of mediating *loxP* site-specific recombination in both ES cells and transgenic mice. The site-specific recombinase Cre can also be used in a predefined cell lineage or at a certain stage of development. See, for example, Gu, H. et al., *Science* 265:103-106, 1994, in which a DNA polymerase  $\beta$  gene segment was deleted from T cells; see also Tsien, J.Z. et al., *Cell* 87:1317-1326, 1996, in which Cre/*loxP* recombination was restricted to cells in the mouse forebrain.) The impact of the mutation on these cells can then be analyzed.

The Cre recombinase catalyzes recombination between 34 bp *loxP* recognition sequences (Sauer, B. and Henderson, N., *Proc. Natl. Acad. Sci. USA* 85:5166-5170, 1988). The *loxP* sequences can be inserted into the genome of embryonic stem cells by homologous recombination such that they flank one or more exons of a gene of interest (making a "floxed" gene). It is crucial that the insertions do not interfere with normal expression of the gene. Mice homozygous for the floxed gene are generated from these embryonic stem cells by conventional techniques and are crossed to a second mouse that harbors a Cre transgene under the control of a tissue type- or cell type-specific transcriptional promoter. In progeny that are homozygous for the floxed gene and that carry the Cre transgene, the floxed gene will be deleted by Cre/*loxP* recombination, but only in those cell types in which the Cre gene-associated promoter is active.

A gene that encodes a protein which acts to have the effect of mimicking the phenotype caused by mutations in a vein-specific or artery-specific gene can



also be used to achieve the same effect as knockouts in vein-specific or artery-specific genes.

A mutation in a gene which encodes a product which prevents binding of ligand to receptor or prevents the functional consequences of such binding and thereby  
5 duplicates the phenotype of a vein- or artery-specific gene knockout (e.g., a dominant negative mutant) can be used as an alternative to a knockout. The mutated gene can be put under the control of a tissue-specific  
10 promoter to be expressed in vein or artery, depending on the tissue-specific gene product whose function is to be inhibited.

In addition, one or more dominant negative alleles of an artery-specific or vein-specific gene can be put  
15 under the control of an inducible promoter so that upon induction, the effect of the inhibition of gene function can be studied. A dominant negative mutant can be isolated or constructed by mutagenesis and methods to make a transgenic mouse.

20 Testing to identify the desired mutant or wild type alleles, or for the identification of other alleles, can be done by PCR on isolated genomic DNA, using appropriate primers, or by Southern blots using appropriate hybridization probes, by a combination of  
25 these procedures, or by other methods.

In addition to the uses of an indicator mouse described in the Examples herein, one use of a mouse having an indicator gene which can mark artery cells is a method for testing an effect of a drug on growth of  
30 arteries. The method can comprise administering the drug to a mouse (e.g., embryo, neonate, juvenile, adult, a wound site, tumor, ischemic lesion or arteriovenous malformation in any of the preceding) having an indicator gene inserted in a gene specifically expressed  
35 in arteries, and observing the effect of the drug on the growth of the arteries, compared to the effect in a

suitable control mouse having an indicator gene, not treated with the drug, but maintained under identical conditions. Similar tests may be performed on an indicator mouse having an indicator gene which marks  
5 vein cells. The effect of the drug can be, for example, to promote growth, to inhibit growth, or to promote aberrant growth. Administration of the drug can be by any suitable route known to those of skill in the art.

An indicator mouse having an indicator gene  
10 inserted in a gene specifically expressed in artery cells can be crossed with a mouse of another strain carrying a mutation in a gene which is to be tested for its effect on the growth and development of blood vessels, to allow for easier visualization of the  
15 effects of the mutation specifically on artery cells. In tests similar to those described above, the effect of a drug can be assessed on the mouse which results from this type of cross, to see, for example, whether the effect of the mutation can be alleviated by the drug.  
20 In like manner, an indicator mouse having an indicator gene inserted in a gene specifically expressed in vein cells can be used in a cross with a mouse with a mutation whose effect on growth of veins is to be evaluated, and the resulting hybrid used in studies of  
25 the growth of veins.

As a result of the work described herein, it is possible to differentiate between arterial endothelial cells (arteries) and venous endothelial cells (veins) by taking advantage of the presence of an artery-specific  
30 or vein-specific gene product on the surface of the cells. Arterial endothelial cells and venous endothelial cells can each be isolated from cells of other tissue types by, for instance, excision of artery or vein tissue from a sample of mammalian tissue,  
35 dissociation of the cells, allowing the cells to bind, under appropriate conditions, to a substance which has

some property or characteristic (e.g., a molecule which provides a label or tag, or molecule that has affinity for both the an artery-specific cell surface protein and another type of molecule) that facilitates separation of  
5 cells bound to the substance from cells not bound to the substance. Separation of the cells can take advantage of the properties of the bound substance. For example, the substance can be an antibody (antiserum, polyclonal or monoclonal) which has been raised against the protein  
10 specific to arterial endothelial cells (or to a sufficiently antigenic portion of the protein) and labeled with a fluorochrome, with biotin, or with another label. Separation of cells bound to the substance can be by fluorescence activated cell sorting  
15 (FACS), for a fluorescent label, by streptavidin affinity column, for a biotin label, by other affinity-based separation methods, or, for example, by antibody-conjugated magnetic beads or solid supports. "Isolated" as used herein for cells indicates that the cells have  
20 been separated from other cell types so as to be a population enriched for a certain cell type, compared to the starting population, and is not limited to the case of a population containing 100% one cell type.

Other means of separation can exploit, for blood  
25 vessel cells bearing an indicator insertion in a gene encoding an artery- or vein-specific protein, the properties of the indicator gene product or portion of fusion protein encoded by the indicator insertion. For example, cells producing an artery- or vein-specific  
30 fusion protein with a green fluorescent protein portion or a blue fluorescent protein portion can be separated from non-fluorescent cells by a cell sorter. Cells producing a fusion protein having an artery- or vein-specific protein portion and an indicator protein or  
35 portion with binding or enzymatic activity can be detected by the ability of the fusion protein to bind to a fluorescent substrate, for example a substrate for  $\beta$ -

galactosidase or  $\beta$ -lactamase, or to produce a fluorescent product in cells.

The isolation of arterial endothelial cells and the isolation of venous endothelial cells allows for tests of these cell types in culture to assess the effects of various drugs, growth factors, ligands, cytokines, members of the Eph and Ephrin families of receptors and ligands, molecules that bind to cell surface proteins, or other molecules which can have effects on the growth and development of arteries and veins. One or more of these substances can be added to the culture medium, and the effects of these additions can be assessed (e.g., by measurements of growth rate or viability, enzyme assays, assays for the presence of cell surface components, incorporation of labeled precursors into macromolecules such as DNA, RNA or proteins).

Isolated arterial endothelial cells or isolated venous endothelial cells can be maintained in artificial growth medium, and an immortalized cell line can be produced from each such isolated cell type (i.e., "transformation") by infection with one of any number of viruses (e.g., retroviruses, by transduction of immortalizing oncogenes such as v-myc or SV40 T antigen) known to effectively transform cells in culture. The virus can be chosen for its species specificity of infectivity (e.g., murine ecotropic virus for mouse cells; amphotropic or pseudotyped viruses for human cells). As an alternative to viral transformation, cells can be maintained in culture by propagating the cells in medium containing one or more growth factors.

Immortalized cell lines derived from either vein or artery cells can be used to produce cDNA libraries to facilitate study of genes actively expressed in each of these tissues. Further, such cell lines can be used to isolate and identify proteins expressed in the cells,

for instance, by purifying the proteins from conditioned growth medium or from the cells themselves.

As one alternative to using immortalized cell lines of arterial or venous origin, cells or cell lines of  
5 non-arterial origin or non-venous origin (e.g.,  
endothelial cells from other tissues, or fibroblasts)  
can be genetically altered (by the introduction of one  
or more non-endogenously expressed genes) to express an  
artery-specific or vein-specific cell surface protein,  
10 and used in methods to detect and identify substances  
that interfere with receptor-ligand interaction.

Introduction of one or more genes into a cell line  
can be, for instance, by transformation, such as by  
electroporation, by calcium phosphate, DEAE-dextran, or  
15 by liposomes, using a vector which has been constructed  
to have an insertion of one or more genes. See Ausubel,  
F.M. et al, *Current Protocols in Molecular Biology*,  
chapter 9, containing supplements through Supplement 40,  
Fall, 1997, John Wiley & Sons, New York. The  
20 introduction of one or more genes to be expressed in a  
cell line can also be accomplished by viral infection,  
for example, by a retrovirus. Retroviral gene transfer  
has been used successfully to introduce genes into whole  
cell populations, thereby eliminating problems  
25 associated with clonal variation.

The ability to differentiate and to isolate the  
cells of veins and arteries allows for a wide variety of  
applications for a wide variety of purposes. For  
example, it is now possible to assess the effects of  
30 various agents, such as drugs, diagnostic reagents and  
environmental/dietary factors, on arteries and veins and  
to determine if the effects observed are common to both  
types of cells or specific to one cell type.

For example, it can no longer be assumed that  
35 angiogenic and anti-angiogenic factors or drugs act  
equivalently on arterial and venous cells. Isolation of

these cell types of these tissues, which is made possible by the present work, allows testing of these angiogenic and anti-angiogenic factors for arterial or venous specificity, which will provide more selective clinical indications for these drugs. It will also allow the discovery of new artery- or vein-selective drugs, such as by high-throughput screening of immortalized arterial or venous endothelial cell lines. Existing drugs can also be selectively targeted to arteries or veins by using the proteins described herein as targeting devices (e.g., liposomes or viral vehicles having the protein or an extracellular domain portion thereof on the viral surface) to deliver drugs (e.g., chemically coupled drugs) to one type of vessel or the other. For example, artery-specific agents can be used to promote collateral growth of arteries to bypass coronary artery occlusions or ischemic lesions.

There are numerous approaches to screening agents for their selective effects (angiogenic or anti-angiogenic, affecting vasotension, or inhibiting formation of atherosclerotic plaques) on arteries and veins. For example, high-throughput screening of compounds or molecules can be carried out to identify agents or drugs which act selectively on arteries or veins or, in some cases, on both. Test agents to be assessed for their effects on artery or vein cells can be any chemical (element, molecule, compound), made synthetically, made by recombinant techniques or isolated from a natural source. For example, test agents can be peptides, polypeptides, peptoids, sugars, hormones, or nucleic acid molecules, such as antisense nucleic acid molecules. In addition, test agents can be small molecules or molecules of greater complexity made by combinatorial chemistry, for example, and compiled into libraries. These libraries can comprise, for example, alcohols, alkyl halides, amines, amides, esters, aldehydes, ethers and other classes of organic

compounds. Test agents can also be natural or genetically engineered products isolated from lysates or growth media of cells -- bacterial, animal or plant -- or can be the cell lysates or growth media themselves.

- 5 Presentation of test compounds to the test system can be in either an isolated form or as mixtures of compounds, especially in initial screening steps.

The compounds or molecules (referred to collectively as agents or drugs) which are screened can  
10 be those already known to have angiogenic, anti-angiogenic activity, anti-plaque activity or vasoactivity, or those of unknown effectiveness. In the case of those agents of known effect, the screening will be useful to identify those drugs which act selectively  
15 on arterial endothelial cells or on venous endothelial cells. In the case of those agents of unknown effect, screening will be useful to newly identify drugs which have angiogenic, antiangiogenic activity, anti-plaque activity or vasoactivity, and to establish the cell type  
20 (arterial, venous) on which they act. For example, immortalized cell lines of arterial or venous origin can be used to screen libraries of compounds to identify drugs with artery- or vein- specific drug effects.

In one embodiment, an assay can be carried out to  
25 screen for drugs that specifically inhibit binding of an Ephrin ligand to its Eph receptor, such as binding of EphrinB2 to the EphB4 receptor, or vice-versa, by inhibition of binding of labeled ligand- or receptor-Fc fusion proteins to immortalized cells. Alternatively,  
30 such libraries can be screened to identify members which enhance binding of an Ephrin ligand to its Eph receptor by enhancing binding of labeled ligand- or receptor-Fc fusion proteins to immortalized cells. Drugs identified through this screening can then be tested in animal  
35 models (e.g., models of cancer, arteriovenous

malformations or coronary artery disease) to assess their activity *in vivo*.

A drug that inhibits interaction of an artery-specific cell surface molecule (e.g., an arterial  
5 endothelial cell-specific surface molecule) with a vein-specific cell surface molecule (e.g., a venous endothelial cell-specific surface molecule) can be identified by a method in which, for example, the  
10 arterial endothelial cell-specific surface molecule and the venous endothelial cell-specific surface molecule are combined with a drug to be assessed for its ability to inhibit interaction between the cell-specific  
15 molecules, under conditions appropriate for interaction between the cell-specific molecules. The cell-specific molecules may be used in the assay such that both are found on intact cells in suspension (e.g., isolated arterial or venous endothelial cells, immortalized cells derived from these, or cells which have been modified to express an artery- or vein-specific cells surface  
20 molecule); one cell type is fixed to a solid support, and the other molecule specific to the other cell type is in soluble form in a suitable solution; or the molecule specific to one cell type is fixed to a solid support while the molecule specific to the other cell  
25 type is found free in a solution that allows for interaction of the cell-specific molecules. Other variations are possible to allow for the convenient assessment of the interaction between the two different cell-specific molecules.

30 In further steps of the assay, the extent to which the cell-specific molecules interact is determined, in the presence of the drug, and in a separate test (control), in the absence of the drug. The extent to which interaction of the cell-specific molecules occurs



in the presence and in the absence of the drug to be assessed is compared. If the extent to which interaction of the cell-specific molecules occurs is less in the presence of the drug than in the absence of the drug, the drug is one which inhibits interaction of the arterial endothelial cell-specific molecule with the venous endothelial cell-specific molecule. If the extent to which interaction of the cell-specific molecules occurs is greater in the presence of the drug than in the absence of the drug, the drug is one which enhances interaction of the arterial endothelial cell-specific molecule with the venous endothelial cell-specific molecule.

In one embodiment of an assay to identify a substance that interferes with interaction of two cell surface molecules, one specific to artery and the other specific to vein (e.g., binding of a ligand to a receptor that recognizes it; interaction between adhesion proteins; interaction between a cell surface protein and a carbohydrate moiety on a cell surface), samples of cells expressing one type of cell surface molecule (e.g., cells expressing an Eph receptor, such as a vein-derived cell line or other cells genetically manipulated to express the Eph receptor) are contacted with either labeled ligand (e.g., an ephrin ligand, a soluble portion thereof, or a soluble fusion protein such as a fusion of the extracellular domain and the Fc domain of IgG) or labeled ligand plus a test compound or group of test compounds. The amount of labeled ligand which has bound to the cells is determined. A lesser amount of label (where the label can be, for example, a radioactive isotope, a fluorescent or colormetric label) in the sample contacted with the test compound(s) is an indication that the test compound(s) interferes with binding. The reciprocal assay using cells expressing a ligand (e.g., an Ephrin ligand or a soluble form

thereof) can be used to test for a substance that interferes with the binding of a receptor or soluble portion thereof.

An assay to identify a substance which interferes with interaction between artery-specific and vein-specific cell surface protein can be performed with the component (e.g., cells, purified protein, including fusion proteins and portions having binding activity) which is not to be in competition with a test compound, linked to a solid support. The solid support can be any suitable solid phase or matrix, such as a bead, the wall of a plate or other suitable surface (e.g., a well of a microtiter plate), column pore glass (CPG) or a pin that can be submerged into a solution, such as in a well. Linkage of cells or purified protein to the solid support can be either direct or through one or more linker molecules.

Upon the isolation from a mammal of a gene expressing an artery-specific or a vein-specific protein, the gene can be incorporated into an expression system for production of a recombinant protein or fusion protein, followed by isolation and testing of the protein in vitro. The isolated or purified protein can also be used in further structural studies that allow for the design of agents which specifically bind to the protein and can act as agonists or antagonists of the receptor or ligand activity of the protein.

In one embodiment, an isolated or purified artery-specific or vein-specific protein can be immobilized on a suitable affinity matrix by standard techniques, such as chemical cross-linking, or via an antibody raised against the isolated or purified protein, and bound to a solid support. The matrix can be packed in a column or other suitable container and is contacted with one or more compounds (e.g., a mixture) to be tested under conditions suitable for binding of the compound to the

protein. For example, a solution containing compounds can be made to flow through the matrix. The matrix can be washed with a suitable wash buffer to remove unbound compounds and non-specifically bound compounds.

- 5 Compounds which remain bound can be released by a suitable elution buffer. For example, a change in the ionic strength or pH of the elution buffer can lead to a release of compounds. Alternatively, the elution buffer can comprise a release component or components designed  
10 to disrupt binding of compounds (e.g., one or more ligands or receptors, as appropriate, or analogs thereof which can disrupt binding or competitively inhibit binding of test compound to the protein).

- Fusion proteins comprising all of, or a portion of,  
15 an artery-specific or a vein-specific protein linked to a second moiety not occurring in that protein as found in nature can be prepared for use in another embodiment of the method. Suitable fusion proteins for this purpose include those in which the second moiety  
20 comprises an affinity ligand (e.g., an enzyme, antigen, epitope). The fusion proteins can be produced by the insertion of a gene specifically expressed in artery or vein cells or a portion thereof into a suitable expression vector, which encodes an affinity ligand.  
25 The expression vector can be introduced into a suitable host cell for expression. Host cells are disrupted and the cell material, containing fusion protein, can be bound to a suitable affinity matrix by contacting the cell material with an affinity matrix under conditions  
30 sufficient for binding of the affinity ligand portion of the fusion protein to the affinity matrix.

- In one aspect of this embodiment, a fusion protein can be immobilized on a suitable affinity matrix under conditions sufficient to bind the affinity ligand  
35 portion of the fusion protein to the matrix, and is contacted with one or more compounds (e.g., a mixture) to be tested, under conditions suitable for binding of

compounds to the receptor or ligand protein portion of the bound fusion protein. Next, the affinity matrix with bound fusion protein can be washed with a suitable wash buffer to remove unbound compounds and non-specifically bound compounds without significantly disrupting binding of specifically bound compounds. Compounds which remain bound can be released by contacting the affinity matrix having fusion protein bound thereto with a suitable elution buffer (a compound elution buffer). In this aspect, compound elution buffer can be formulated to permit retention of the fusion protein by the affinity matrix, but can be formulated to interfere with binding of the compound(s) tested to the receptor or ligand protein portion of the fusion protein. For example, a change in the ionic strength or pH of the elution buffer can lead to release of compounds, or the elution buffer can comprise a release component or components designed to disrupt binding of compounds to the receptor or ligand protein portion of the fusion protein (e.g., one or more ligands or receptors or analogs thereof which can disrupt binding of compounds to the receptor or ligand protein portion of the fusion protein).

Immobilization can be performed prior to, simultaneous with, or after contacting the fusion protein with compound, as appropriate. Various permutations of the method are possible, depending upon factors such as the compounds tested, the affinity matrix selected, and elution buffer formulation. For example, after the wash step, fusion protein with compound bound thereto can be eluted from the affinity matrix with a suitable elution buffer (a matrix elution buffer). Where the fusion protein comprises a cleavable linker, such as a thrombin cleavage site, cleavage from the affinity ligand can release a portion of the fusion with compound bound thereto. Bound compound can then be

released from the fusion protein or its cleavage product by an appropriate method, such as extraction.

One or more compounds can be tested simultaneously according to the method. Where a mixture of compounds is tested, the compounds selected by the foregoing processes can be separated (as appropriate) and identified by suitable methods (e.g., PCR, sequencing, chromatography). Large combinatorial libraries of compounds (e.g., organic compounds, peptides, nucleic acids) produced by combinatorial chemical synthesis or other methods can be tested (see e.g., Ohlmeyer, M.H.J. et al., *Proc. Natl. Acad. Sci. USA* 90:10922-10926 (1993) and DeWitt, S.H. et al., *Proc. Natl. Acad. Sci. USA* 90:6909-6913 (1993), relating to tagged compounds; see also, Rutter, W.J. et al., U.S. Patent No. 5,010,175; Huebner, V.D. et al., U.S. Patent No. 5,182,366; and Geysen, H.M., U.S. Patent No. 4,833,092). Where compounds selected from a combinatorial library by the present method carry unique tags, identification of individual compounds by chromatographic methods is possible. Where compounds do not carry tags, chromatographic separation, followed by mass spectrophotometry to ascertain structure, can be used to identify individual compounds selected by the method, for example.

An *in vivo* assay useful to identify drugs which act selectively on arteries or on veins is also available. It is carried out using transgenic animals, such as those described herein, which make it possible to visualize angiogenic processes. For example, an EphrinB2 knockout mouse containing a marker, such as a *tau-lacZ* insertion, that marks all arteries but not veins, can be used for a variety of *in vivo* assays. Other marker genes that can be used, for instance, are genes expressing alkaline phosphatase, blue fluorescent

protein or green fluorescent protein. The mouse, or the targeted allele it contains, can be used to study angiogenic processes, such as tumor angiogenesis and ischemia-associated cardiac neovascularization, in  
5 arteries, independent of veins. For example, tumor cells can be implanted in the indicator mouse and arterial vessel growth into the tumor can be visualized by *lacZ* staining. Alternatively, mice bearing the targeted allele can be crossed with a mouse model of  
10 another condition, such as vascular degeneration or neovascularization, to be visualized. The arterial-specific aspects of the process can be visually monitored by *lacZ* staining. An indicator of this type can also be used to assess drugs for their angiogenic or  
15 anti-angiogenic effects.

A gene product produced specifically by arterial endothelial cells (arteries) and not by other cell types allows for the specific targeting of drugs, diagnostic agents, tagging labels, histological stains or other  
20 substances specifically to arteries. In an analogous manner, a gene product identified as produced specifically by venous endothelial cells (veins) and not detectably produced by other cell types allows for the specific targeting and delivery of drugs, diagnostic  
25 agents, tagging labels, histological stains or other substances specifically to veins. The following description of targeting vehicles, targeted agents and methods is presented using EphrinB2 as an illustration of a gene product produced by arterial endothelial cells  
30 and not by vein cells and EphB4 as an illustration of a gene product produced by venous endothelial cells and not by artery cells. However, this description applies equally well to other artery-specific and vein-specific gene products that can be used to identify these tissue  
35 types.

The differential expression of EphrinB2 in arteries and of Eph4 in veins allows for the specific targeting of drugs, diagnostic agents, imaging agents, or other substances to the cells of arteries or of veins. A targeting vehicle can be used for the delivery of such a substance. Targeting vehicles which bind specifically to EphrinB2 or to Eph4 can be linked to a substance to be delivered to the cells of arteries or veins, respectively. The linkage can be via one or more covalent bonds, or by high affinity non-covalent bonds. A targeting vehicle can be an antibody, for instance, or other compound which binds either to EphrinB2 or to EphB4 with high specificity. Another example is an aqueously soluble polypeptide having the amino acid sequence of the extracellular domain of EphB4, or a sufficient portion of the extracellular domain (or a polypeptide having an amino acid sequence conferring a similar enough conformation to allow specific binding to EphrinB2), which can be used as a targeting vehicle for delivery of substances to EphrinB2 in arteries. Similarly, a soluble polypeptide having the amino acid sequence of the extracellular domain of EphrinB2 or a sufficient antigenic portion of the extracellular domain (or a polypeptide having an amino acid sequence conferring a similar enough conformation to allow specific binding to EphB4), can be used to target substances to EphB4 in veins.

Targeting vehicles specific to an artery-specific Ephrin ligand (e.g., EphrinB2) or to a vein-specific Eph receptor (e.g., EphB4) have in vivo (e.g., therapeutic and diagnostic) applications. For example, an antibody which specifically binds to EphrinB2 can be conjugated to a drug to be targeted to arteries (e.g., a therapeutic, such as an anti-plaque agent).

Alternatively, an antibody which specifically binds to EphB4 can be used to target a drug to veins. A substance (e.g., a radioactive substance) which can be detected (e.g., a label) in vivo can also be linked to a targeting vehicle which specifically binds to an artery-specific Ephrin ligand (e.g., EphrinB2) and the conjugate can be used as a labeling agent to identify arteries. Similarly, a detectable label can be linked to a targeting vehicle which specifically binds a vein-specific Eph receptor (e.g., EphB4) to identify veins.

Targeting vehicles specific to EphrinB2 or to EphB4 find further applications in vitro. For example, an EphB4-specific targeting vehicle, such as an antibody (a polyclonal preparation or monoclonal) which specifically binds to EphB4, can be linked to a substance which can be used as a stain for a tissue sample (e.g., horseradish peroxidase) to provide a method for the identification of veins in a sample. Likewise, an antibody which specifically binds to EphrinB2 or to the extracellular domain of EphrinB2 can be used in the identification of arteries. For instance, in a biopsied tissue sample, as from a tumor, or from an arteriovenous malformation in a child or adult, antibody to EphrinB2 or to the extracellular domain of EphrinB2 can be used to identify artery tissue and to distinguish it from vein tissue.

To treat malformed, painful or cosmetically undesirable veins, an agent which acts against them (e.g., antiangiogenic factors) can be linked to an EphB4-specific vehicle for local administration to the veins. For example, anti-angiogenic factors can be injected into varicose veins.

Targeted agents directed to either an artery-specific Ephrin family ligand (e.g., EphrinB2) or a



vein-specific Eph family receptor (e.g., EphB4) can also be used when it is desired to produce an effect on both arteries and veins. For example, limited amounts of targeted agents comprising an anti-angiogenic drug and a  
5 targeting vehicle to either EphrinB2, EphB4, or both, can be administered locally to sites of angiogenesis, such as sites of tumor formation or sites of undesirable neovascularization where it is desired to inhibit the growth of blood vessels, or to areas in which increased  
10 vascularization is desired to enhance growth or establishment of blood vessels.

Substances that act as agonists or antagonists of an artery-specific Ephrin family ligand (e.g., EphrinB2) or a vein-specific Eph family receptor (e.g., EphB4) can  
15 be used as angiogenic or anti-angiogenic agents. Drugs that target these molecules will selectively influence arterial and venous angiogenesis. For example, monoclonal antibodies to EphrinB2 or EphB4 can serve as artery- or vein-specific angiogenic or anti-angiogenic  
20 agents. Drugs that interfere with EphrinB2 function (for instance, blocking antibodies) can be used in anti-angiogenic methods of therapy. As can be concluded from the phenotype shown by the *EphrinB2<sup>tlacZ</sup>/EphrinB2<sup>tlacZ</sup>* mutant mice, antagonists of EphrinB2 or antagonists of  
25 EphB4 will inhibit angiogenesis. Agents which are agonists of both EphrinB2 and EphB4 will promote angiogenesis.

In another example, soluble agonists which comprise the extracellular domain of an Ephrin family ligand or  
30 the extracellular domain of an Eph family receptor fused to the Fc domain of human IgG can be produced. For example, an EphB4 or an EphrinB2 hybrid protein in which the extracellular domain of the membrane protein is fused to the Fc domain of human IgG can be used (Wang,

H.U. and D.J. Anderson, *Neuron* 18:383-396 (1997)). See, for examples of methods Stein, E. et al., *Genes and Dev.* 12:667-678 (1998), regarding experiments on responses of cells to clustered Ephrin-B1/Fc fusion proteins.

- 5 Clustering of these hybrid molecules with anti-human Fc antibodies generates soluble agonists: Ephrin-derived "ligand-bodies" for Eph receptors, and conversely, Eph-derived "receptor bodies" for Ephrins. Non-clustered forms of these hybrid molecules can be used as
- 10 antagonists.

A further application of isolated arterial endothelial cells and isolated venous endothelial cells is the genetic alteration of the isolated cells and the administration of these cells, preferably intravenously,

15 to the host mammal from which the cells were isolated, or into another compatible host, where the cells can be incorporated into a blood vessel of the appropriate type. In this way, the effects of a genetic defect which is manifested in arteries or in veins can be

20 ameliorated. It has been demonstrated that circulating endothelial cell progenitors can migrate to sites of neovascularization and be incorporated into blood vessels (Asahara et al., *Science* 275:964-967 (1997)).

The introduction of a gene (an endogenous gene that

25 has been altered, or a gene originally isolated from a different organism, for example) into cells can be accomplished by any of several known techniques, for example, by vector mediated gene transfer, as by amphotropic retroviruses, calcium phosphate, or liposome

30 fusion, for example.

A gene intended to have an effect on arteries or veins in a host mammal can be delivered to isolated artery cells or isolated vein cells by the use of viral vectors comprising one or more nucleic acid sequences

encoding the gene of interest. Generally, the nucleic acid sequence has been incorporated into the genome of the viral vector. In vitro, the viral vector containing the nucleic acid sequences encoding the gene can be

5 contacted with a cell and infection can occur. The cell can then be used experimentally to study, for example, the effect of the gene on growth of artery or vein cells in vitro or the cells can be implanted into a patient for therapeutic use. The cells to be altered by

10 introduction or substitution of a gene can be present in a biological sample obtained from the patient and used in the treatment of disease, or can be obtained from cell culture and used to dissect developmental pathways of arteries and veins in in vivo and in vitro systems.

15 After contact with the viral vector comprising a nucleic acid sequence encoding the gene of interest, the treated artery or vein cells can be returned or readministered to a patient according to methods known to those practiced in the art. Such a treatment

20 procedure is sometimes referred to as ex vivo treatment. Ex vivo gene therapy has been described, for example, in Kasid, et al., *Proc. Natl. Acad. Sci. USA* 87:473 (1990); Rosenberg, et al., *New Engl. J. Med.* 323:570 (1990); Williams, et al., *Nature* 310:476 (1984); Dick, et al.,

25 *Cell* 42:71 (1985); Keller, et al., *Nature* 318:149 (1985) and Anderson, et al., U.S. Patent No. 5,399,346 (1994).

Generally, viral vectors which can be used therapeutically and experimentally are known in the art. Examples include the vectors described by Srivastava,

30 A., U.S. Patent No. 5,252,479 (1993); Anderson, W.F., et al., U.S. Patent No. 5,399,346 (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley & Sons, Inc. (1998). Suitable viral vectors for the delivery of nucleic acids to cells include, for example,

replication defective retrovirus, adenovirus, parvovirus (e.g., adeno-associated viruses), and coronavirus.

Examples of retroviruses include avian leukosis-sarcoma, mammalian C-type, B-type viruses, lentiviruses (Coffin,

- 5 J.M., "Retroviridae: The Viruses and Their Replication", In: *Fundamental Virology*, Third Edition, B.N. Fields, et al., eds., Lippincott-Raven Publishers, Philadelphia, PA, (1996)). The mechanism of infectivity depends upon the viral vector and target cell. For example,
- 10 adenoviral infectivity of HeLa cells occurs by binding to a viral surface receptor, followed by receptor-mediated endocytosis and extrachromosomal replication (Horwitz, M.S., "Adenoviruses" In: *Fundamental Virology*, Third Edition, B.N. Fields, et al., eds., Lippincott-
- 15 Raven Publishers, Philadelphia, PA, (1996)).

The present invention is illustrated by the following examples, which are not intended to be limiting in any way.

## EXAMPLES

## Experimental Procedures

The following experimental procedures were used in the examples which follow.

- 5 Targeted disruption of the EphrinB2 gene. A 200 base pair probe starting from the ATG of the mouse EphrinB2 gene (Bennett, B. D., et al., *Proc. Natl. Acad. Sci. USA* 92:1866-1870 (1995)) was used to screen a 129SVJ genomic library (Stratagene). Analysis of
- 10 several overlapping clones revealed that the first exon, including the signal sequence, ends at 131 base pairs after the ATG. Further phage analysis and library screens revealed that the rest of the EphrinB2 gene was located at least 7 kb downstream from the first exon.
- 15 To construct a targeting vector (Fig. 1B), a 3kb XbaI-NcoI fragment whose 3' end terminated at the ATG was used as the 5' arm. A 5.3 kb Tau-lacZ coding sequence (Mombaerts, P., et al., *Cell* 87:675-686 (1996)) was fused in frame after the ATG. The PGKneo gene (Ma, Q., et al., *Neuron* 20:469-482 (1998)) was used to replace a
- 20 2.8 kb intronic sequence 3' to the first exon. Finally, a 3.2 kb downstream EcoRI-EcoRI fragment was used as the 3' arm. Normal (6kb) and targeted (9kb) loci are distinguished by HindIII digestion when probed with a
- 25 1kb HindIII-XbaI genomic fragment. Electroporation, selection and blastocyst-injection of AB-1 ES cells were performed essentially as described Ma, Q., et al. (*Neuron* 20:469-482 (1998)), with the exception that FIAU-selection was omitted. ES cell targeting
- 30 efficiency via G418 selection was 1 out of 18 clones. Germline transmission of the targeted EphrinB2 locus (Fig. 1C) in heterozygous males was confirmed by Southern blotting of tail DNA of adult mice, using a 1kb HindIII-

XbaI probe. Subsequent genotyping was done by genomic PCR. Primers for Neo are

5'-AAGATGGATTGCACGCAGGTTCTC-3' (SEQ ID NO.: 1) and  
5'-CCTGATGCTCTTCGTCCAGATCAT-3' (SEQ ID NO.: 2). Primers  
5 for the replaced intronic fragment are  
5'-AGGACGGAGGACGTTGCCACTAAC-3' (SEQ ID NO.: 3) and  
5'-ACCACCAGTTCCGACGCGAAGGGA-3' (SEQ ID NO.: 4).

LacZ, PECAM-1, and histological staining. Embryos  
and yolk sacs were removed between E7.5 and E10.0, fixed  
10 in cold 4% paraformaldehyde/PBS for 10 minutes, rinsed  
twice with PBS, and stained for 1 hour to overnight at  
37°C in X-gal buffer (1.3 mg/ml potassium ferrocyanide,  
1 mg/ml potassium ferricyanide, 0.2% Triton X-100, 1 mM  
MgCl<sub>2</sub>, and 1 mg/ml X-gal [5-bromo-4-chloro-3-indolyl-β-D-  
15 galactopyranoside] in PBS, pH 7.2). LacZ stained  
embryos were post-fixed and photographed, or sectioned  
on a cryostat after embedding in 15% sucrose and 7.5%  
gelatin in PBS. Procedures for whole mount or section  
staining with anti-PECAM-1 antibody (clone MEC 13.3,  
20 Pharmingen) were done essentially as described Ma et al.  
(*Neuron* 20:469-482 (1998); Fong et al., *Nature* 376:66-70  
(1995)). Horseradish peroxidase-conjugated secondary  
antibodies were used for all PECAM-1 stainings. LacZ-  
stained yolk sacs were sectioned in gelatin and then  
25 subjected to hematoxylin counter-staining by standard  
procedures.

In situ hybridization. In situ hybridization on  
frozen sections was performed as previously described  
(Birren et al. *Development* 119:597-610 (1993)). Whole-  
30 mount in situ hybridization followed a protocol by  
Wilkinson, D.G., (Whole-mount in situ hybridization of  
vertebrate embryos. pp. 75-83 In: *In Situ Hybridization:  
A Practical Approach* (ed. D.G. Wilkinson) IRL Press,  
Oxford :75-83 (1992). Bluescript vectors (Stratagene)  
35 containing cDNAs for EphB2/Nuk and EphB4/Myk-1 were

generated as described Wang, H.U. and Anderson, D.J.  
(*Neuron* 18:383-396 (1997)).

Example 1: Targeted mutagenesis of *EphrinB2* in mice.

Targeted disruption of the *EphrinB2* gene was  
5 achieved by homologous recombination in embryonic stem  
cells. The targeting strategy involved deleting the  
signal sequence and fusing a tau-lacZ indicator gene in  
frame with the initiation codon. The expression pattern  
of  $\beta$ -galactosidase in heterozygous (*EphrinB2*<sup>lacZ/+</sup>)  
10 embryos was indistinguishable from that previously  
reported for the endogenous gene. (Bennett, B.D. et al.  
*Proc. Natl. Acad. Sci. USA* 92: 1866-1870 (1995);  
Bergemann, A.D. et al. *Mol. Cell. Bio.* 1995:4921-4929  
(1995); Wang, H.U. and Anderson, D.J. *Neuron* 18:383-396  
15 (1997)). While prominent expression was detected in the  
hindbrain and somites, lower levels were observed in the  
aorta and heart as early as E8.25. Expression in the  
yolk sac was first detected at E8.5. Heterozygous  
animals appeared phenotypically normal. In homozygous  
20 embryos, growth retardation was evident at E10 and  
lethality occurred with 100% penetrance around E11. No  
expression of endogenous *EphrinB2* mRNA was detected by  
in situ hybridization, indicating that the mutation is a  
null. Somite polarity, hindbrain segmentation, and the  
25 metameric patterning of neural crest migration (in which  
*EphrinB2* and related ligands have previously been  
implicated Xu, W. et al. *Development* 121:4005-4016  
(1995); Wang, H.U. and Anderson, D.J. *Neuron* 18:383-396  
(1997); Krull, C.E. et al. *Curr. Biol.* 7:571-580 (1997);  
30 Smith, A. et al. *Curr. Biol.* 7:561-570 (1997)) appeared  
grossly normal in homozygous mutant embryos.

Example 2: Reciprocal expression pattern of EphrinB2 and EphB4 in arteries and veins.

The enlarged heart observed in dying mutant embryos prompted examination of the expression of *EphrinB2<sup>tlacZ</sup>* in the vascular system in detail. Expression was consistently observed in arteries but not veins. In the yolk sac, for example, the posterior vessels connected to the vitelline artery, but not the vitelline vein, expressed the gene, as detected by lacZ staining. In the trunk, labeling was detected in the dorsal aorta, vitelline artery, umbilical artery and its allantoic vascular plexus, but not the umbilical, anterior and common cardinal veins (the umbilical vein is labeled with anti-PECAM-1 antibody). In the head, labeling was seen in branches of the internal carotid artery, but not in those of the anterior cardinal vein. In situ hybridization with *EphrinB2* cDNA probes confirmed that the selective expression of *tau-lacZ* in arteries correctly reflected the pattern of expression of the endogenous gene. Examination of the expression of the four *EphB* family genes, as well as Eph A4/Sek1, which are receptors for EphrinB2 Gale, N.W. et al., Neuron 17:9-19 (1996) revealed complementary expression of EphB4 in veins but not arteries, including the vitelline vein and its branches in anterior portion of the yolk sac.

Example 3: Vasculogenesis occurs normally in EphrinB2 mutant embryos.

The formation of the major vessels in the trunk was unaffected by the lack of EphrinB2, as examined by lacZ and PECAM-1 double staining of 9 somite embryos. Expression of *EphrinB2-lacZ* was seen in the dorsal aorta and vitelline artery, but not the umbilical and posterior cardinal veins. The dorsal aorta, vitelline



artery, posterior cardinal and umbilical veins, for example, formed, although some dilation and wrinkling of the vessel wall was observed. Similarly the intersomitic vessels originating from the dorsal aorta formed at this stage. Between E8.5 and E9.0, the primitive endocardium appeared only mildly perturbed in mutants, while a pronounced disorganization was apparent at E10. Red blood cells developed and circulated normally up to E9.5 in both the mutant yolk sac and embryo proper.

Example 4: Extensive intercalation of yolk sac arteries and veins revealed by EphrinB2 expression.

In the yolk sac, the vitelline artery and its capillary network occupy the posterior region, and the vitelline vein and its capillaries the anterior region. At E8.5, a stage at which the primary capillary plexus has formed but remodeling has not yet occurred, asymmetric expression of *EphrinB2- $\tau$ lacZ* in heterozygous embryos was evident at the interface between the anterior and posterior regions. Apparently homotypic remodeling of  $\beta$ -galactosidase<sup>+</sup> arterial capillaries into larger, branched trunks clearly segregated from venous vessels was evident between E9.0 and E9.5. At this stage, expression of the receptor EphB4 was clearly visible on the vitelline veins but not arteries. Thus arterial and venous endothelial capillaries are already molecularly distinct following vasculogenesis and prior to angiogenesis.

While textbook diagrams (Carlson, B.M Patten's *Foundations of Embryology* (1981)) of the yolk sac capillary plexus depict a non-overlapping boundary between the arterial and venous capillary beds, expression of *EphrinB2- $\tau$ lacZ* allowed detection of a previously-unrecognized extensive intercalation between arteries and veins across the entire anterior-posterior

extent of the yolk sac; this was observed in the heterozygote, but not in the homozygote. Double-labeling for PECAM and  $\beta$ -galactosidase revealed that the interface between the arteries and veins occurs between microvessel extensions that bridge larger vessels interdigitating en passant.

Example 5: Disrupted angiogenesis in the yolk sac of *EphrinB2<sup>tlacZ</sup>*/*EphrinB2<sup>tlacZ</sup>* embryos.

Defects in yolk sac angiogenesis were apparent by E9.0 and obvious at E9.5. There was an apparent block to remodeling at the capillary plexus stage, for both arterial vessels as revealed by  $\beta$ -galactosidase staining and venous vessels in the anterior region of the sac as revealed by PECAM staining. Thus, disruption of the *EphrinB2* ligand gene caused both a non-autonomous defect in EphB4 receptor-expressing venous cells, and an autonomous defect in the arteries themselves.

This defect was accompanied by a failure of intercalating bi-directional growth of arteries and veins across the antero-posterior extent of the yolk sac, so that an interface between *EphrinB2*-expressing and non-expressing zones at the midpoint of the sac was apparent. (However, small patches of *lacZ* expression were occasionally visible within the anterior venous plexus, suggesting that some arterial endothelial cells may have become incorporated into venous capillaries.) These observations imply a close relationship between the remodeling of the capillary plexus into larger vessels and the intercalating growth of these vessels. The large  $\beta$ -galactosidase<sup>+</sup> vitelline arteries as well as vitelline veins present at the point of entry to the yolk sac of the embryo-derived vasculature appeared unperturbed in the mutant, however. This is consistent with the observation that the mutation does not affect formation of the primary trunk vasculature. It also

argues that the yolk sac phenotype is due to a disruption of intrinsic angiogenesis and is not secondary to a failure of ingrowth of embryo-derived vessels.

5       Histological staining (hematoxylin) of sectioned yolk sacs revealed an accumulation of elongated support cells (mesenchymal cells or pericytes) in close association with the endothelial vessels at E10 and E10.5. In mutant yolk sacs, these support cells  
10 appeared more rounded, suggesting a defect in their differentiation. Moreover, in contrast to heterozygous yolk sacs, where vessels of different diameters began to appear at E9.5 and vessel diameter increased through E10.5, capillary diameter appeared relatively uniform  
15 and did not increase with age in the mutants. At E10.5, arteries appear dilated, as if fusion of vessels occurred without encapsulation by support cells. The mutant capillaries also failed to delaminate from the basal endodermal layer.

20 Example 6: Absence of internal carotid arterial branches and defective angiogenesis of venous capillaries in the head of mutant embryos.

Similar to the yolk sac phenotype, the capillary bed of the head appeared dilated in the mutant, and  
25 apparently arrested at the primary plexus stage. Staining for  $\beta$ -galactosidase revealed that the anterior-most branches of the internal carotid artery failed to develop in the mutant. Unlike the case in the yolk sac, therefore, the malformed capillary beds must be entirely  
30 of venous origin. However the anterior branches of the anterior cardinal vein formed although they were slightly dilated. Taken together, these data indicate that in the head, venous angiogenesis is blocked if the normal interaction with arterial capillaries is  
35 prevented. The angiogenic defects observed in the head and yolk sac are unlikely to be secondary consequences

of heart defects (see below), since they are observed starting at E9.0 and the embryonic blood circulation appears normal until E9.5.

Example 7: EphrinB2-dependent signaling between  
5 endocardial cells is required for myocardial trabeculae formation.

Examination of ligand and receptor expression in wild-type hearts revealed expression in the atrium of both EphrinB2 (as detected by lacZ staining) and EphB4  
10 (as detected by in situ hybridization). Expression of both ligand and receptor was also detected in the ventricle in the endocardial cells lining the trabecular extensions of the myocardium. Double-labeling experiments suggested that the ligand and receptor are  
15 expressed by distinct but partially overlapping cell populations, although the resolution of the method does not permit us to distinguish whether this overlap reflects co-expression by the same cells, or a close association of different cells. In any case, expression  
20 of EphrinB2 and EphB4 does not define complementary arterial (ventricular) and venous (atrial) compartments of the heart, unlike the extra-cardiac vasculature.

Heart defects commenced at E9.5 and were apparent in mutant embryos at E10 both morphologically and by  
25 wholemount PECAM-1 staining. Sections revealed an absence of myocardial trabecular extensions, although strands of EphrinB2-expressing endocardial cells were still visible. Thus, mutation of the ligand-encoding gene caused a non-autonomous defect in myocardial cells,  
30 similar to the effect of a mutation in the *neuregulin-1* gene. (Meyer, D. and Birchmeier, C, *Nature* 378:386-390 (1995)) Paradoxically, however, in this case the EphB4 receptor is expressed not on myocardial cells, as is the case for the *neuregulin-1* receptors *erbB2* and *erbB4* (Lee  
35 et al., *Nature* 378:394-398 (1995); Gassmann, et al.,

Nature 378:390-394 (1995), but rather on endocardial cells. Expression of any of the other receptors for Ephrin B family ligands (Eph B1, B2, B3 and A4) was detected in this tissue. This suggests that in the heart, ligand-receptor interactions among endothelial cells may in turn affect interactions with smooth muscle cells.

Example 8: Ephrin B2 is required for vascularization of the neural tube.

10 In *EphrinB2<sup>tlacZ</sup>/EphrinB2<sup>tlacZ</sup>* embryos capillary ingrowth into the neural tube failed to occur. Instead, EphrinB2-expressing endothelial cells remained associated with the exterior surface of the developing spinal cord. Comparison of  $\beta$ -galactosidase to pan-  
15 endothelial PECAM-1 and EphB4 expression provided no evidence of a separate, venous capillary network expressing EphB4 in the CNS at this early stage (E9-E10). Rather, expression of a different EphrinB2 receptor, Eph B2, was seen in the neural tube as  
20 previously reported Henkemeyer, et al., *Oncogene* 9:1001-1014 (1994), where no gross morphological or patterning defects were detectable. In this case, therefore, the mutation does not appear to cause a non-autonomous phenotype in receptor-expressing cells, rather only an  
25 autonomous effect on ligand-expressing cells.

Example 9: EphrinB2 is artery-specific in adult tissues

To determine whether ephrin-B2 is expressed in adult tissues in an artery-specific manner, we performed histochemical staining for  $\beta$ -galactosidase on  
30 *ephrin-B2<sup>taulacZ</sup>/+* heterozygous mice. Antibody staining for PECAM-1, a pan-endothelial marker, was performed on the same or on adjacent sections to reveal non-arterial vessel (i.e., veins). Ephrin-B2 is expressed throughout

the adult mouse in an artery-specific manner, in tissues including the heart, leg muscle, kidney, liver and fat. Expression was detected in vessels of all diameters, including large arteries, arterioles and the  
5 smallest-diameter capillaries. It had been previously assumed that capillaries by definition have neither arterial nor venous identity. These results show that this is not the case, and that arterial identity extends into the capillary beds.

10 Sections through adult arteries were double labeled by histochemical staining for  $\beta$ -galactosidase (lacZ) to reveal ephrin-B2-taulacZ expression, and with antibody to PECAM-1 as a pan-endothelial marker. LacZ (blue from X-gal) staining revealed ephrinB2 expression in dorsal  
15 aorta but not in inferior vena cava, in femoral artery next to the leg bone, but not in femoral vein, and in coronary epicardial artery, but not in coronary vein.

Similar staining of other sections revealed the presence of EphrinB2 in kidney arteriole, liver  
20 arteriole and small muscle arteriole, as well as arterial capillary. Kidney venule, hepatic vein, and muscle veins were lacZ-negative but PECAM-1 positive.

Gut fat was stained for lacZ ( $\beta$ -galactosidase) and labeled with PECAM-1 to reveal venous vessels as well as  
25 arterial vessels. EphrinB2 is expressed by arterioles and arterial capillaries and in arteriole but not in PECAM-1 positive venule. A further section showed that EphrinB2 is expressed by arterial capillaries surrounded by PECAM-1 positive non-arterial capillaries.

30 Example 10: Ephrin-B2 is expressed during tumor angiogenesis

It had been assumed that tumor vessels sprout from the post-capillary venules. To address the question of whether EphrinB2 is expressed during tumor angiogenesis,

Lewis Lung Carcinomas were implanted subcutaneously in the dorsal region of *ephrin-B2<sup>taulacZ</sup>/+* heterozygous females. After one week, the tumors were removed and processed for  $\beta$ -galactosidase histochemistry in whole  
5 mounts. The results indicate clearly that ephrin-B2 is in fact expressed by tumor vessels. This was confirmed by double-staining of sections cut through such tumors, for  $\beta$ -galactosidase and PECAM-1.

EphrinB2 expression by tumor capillaries. Lewis  
10 Lung Carcinoma (LLC) cells were implanted subcutaneously in the dorsal region of *EphrinB2-taulacZ* heterozygous females. After one week, tumors were removed for staining. EphrinB2 positive arterial capillaries were observed in peripheral tumor tissue. Double labeling  
15 with PECAM-1 colocalized the EphrinB2 lacZ (staining blue) and PECAM-1 (staining brown) signals in arterial capillaries, and reveals non-arterial capillaries labeled by PECAM-1 only.

#### Example 11: Screen for clones containing artery- 20 specific genes

A summary of the screening procedures used is provided in Appendix I. Briefly, endothelial cells were isolated from dissociated embryonic yolk sac, vitelline arteries or vitelline veins using positive selection  
25 with antibody to PECAM-1 (magnetic bead or FACS). cDNA was synthesized from lysates of either single cells or small numbers (ca. 200) of cells, and amplified by PCR. To confirm that the cDNAs were from arterial or venous endothelial cells, the cDNA synthesized from each cell  
30 prep was Southern blotted and hybridized in quadruplicate with a series of probes, including tubulin (ubiquitously expressed) the pan-endothelial probes *Flk1* and *Flt1*, and the arterial-specific probe *ephrin-B2*. cDNAs that contained *Flk1*, *Flt1* and *ephrin-B2* sequences

were considered arterial, while those that contained the pan-endothelial markers but not ephrin-B2 were considered venous. Thus the use of ephrin-B2 probes in this procedure was essential to confirm the arterial vs. 5 venous nature of the cDNAs synthesized.

To isolate additional arterial-specific genes from these cDNAs, they were cloned into a phage lambda vector to generate cDNA libraries. Plaques from these libraries were then screened in duplicate filter lifts 10 with arterial- or venous-specific cDNA probes made from either single cells or pools of cells. Plaques exhibiting differential hybridization to the arterial vs. venous probes were isolated, and the inserts were amplified using T3-T7 primers, and re-analyzed by cDNA 15 Southern blotting. Two different pairs of arterial-venous endothelial cell cDNA probes (vitelline artery-vein cells and single yolk sac arterial and venous endothelial cells) were used in the Southern blot. Most of the clones were strongly expressed in 20 arterial cells, and expressed weakly or not detectably by venous cells. The initial screen was designed to isolate additional arterial-specific genes. Twelve candidate arterial-specific clones were isolated using the single-cell probes, while one clone was isolated 25 using the pooled probe. The arterial-specific expression of these genes in vivo can be confirmed by in situ hybridization experiments. Methods such as these can be applied to arterial endothelial cells or vein-specific cells.

30 These data show that it will be possible to isolate novel arterial- or venous-specific genes from single endothelial cells. Such vessel type-restricted genes may provide insights into the physiological differences between arterial and venous endothelial cells. Methods



such as those described herein can also identify genes involved in the etiology of arterial and venous specific diseases, such as arterial hypertension, atherosclerosis, deep venous thrombosis, and certain types of venous malformations. They can also provide candidate genes for human genetic disorders of the circulatory system. The gene products of such genes can then serve as novel drug targets.

#### Appendix I: Single Cell PCR, 3' cDNA Library

- 10 Construction And Differential Screening Procedure to Isolate Novel Arterial- or Venous-Specific Genes
  1. Dissection of vitelline arteries and vitelline veins from E12.5 to E14.5 yolk sacs, based on morphological criteria.
  - 15 2. Dissociation of yolk sacs in collagenase solution (5 mg/ml) at 37°C for 45'.
  3. Isolate single or small groups of endothelial cells (ECs) by one of the following methods.
    - a) Magnetic bead-based separation using PECAM-1 as primary antibody.
    - 20 b) FACS purification using PECAM-1-FITC primary antibody.
    - c) GFP fluorescence from tie2-GFP transgenic mouse for endothelial cell identification, followed by microcapillary mouth-pipeting.
  - 25 4. Lyse the single cells in PCR tubes at 65°C for 1'.
  5. Keep 1-2' at room temperature to allow the oligo-T to anneal to RNA.
  6. Reverse transcription using AMV and MMLV enzyme mixture, at 37°C for 15'.
  - 30 7. Poly-A tailing with terminal transferase and dATP, at 37°C for 15'.
  8. PCR reaction set up:

- 100 µl reaction, 5-fold normal level of dNTP mix,  
high  
concentration of Taq.  
Using a single PCR primer with 24 (T)s at 3' end  
for symmetrical amplification.
- 5 9. cDNA Southern blotting with endothelial specific  
and arterial/venous specific 3' probes on the  
amplified cDNAs for each cell prep.
- 10 10. Choose a few good cells that give strong signals  
for the appropriate probes. Isolate cDNAs from 500  
bp to 2 kb on agarose gel.
11. Precipitate and quantify cDNA.
12. Ligate into lambda ZAPII (Stratagene; LaJolla, CA)  
phage arms for cDNA libraries.
- 15 13. Plate the library at very low density: 1000  
pfu/plate. Take duplicate filter lifts.
14. Screen duplicate filters with probes made from  
vitelline artery cells and vitelline vein cells.
15. Pick differentially expressed phage clones.
- 20 Reverse (cDNA) Southern blot confirmation of phage  
inserts by probing with probes made from vitelline  
artery cells and vitelline vein cells.
16. In situ hybridization to examine the expression  
patterns of cDNA fragments.
- 25 Example 12: Generation of monoclonal antibodies against  
the extracellular domain of ephrin-B2
- While polyclonal rabbit antibodies to fragments of  
ephrin-B2 expressed in bacteria had previously been  
reported, such antibodies are typically not reactive  
30 with native forms of the protein on the cell surface,  
and therefore are not useful for many applications  
including cell sorting, functional inhibition and  
drug-targeting. To generate antibodies with more

desirable binding properties, we expressed the extracellular domain of ephrin-B2 as a glycoposphatidylinositol (GPI)-linked form on Chinese Hamster Ovary (CHO) cells. Hamsters were immunized with  
5 these cells, hybridomas prepared by fusion with mouse myeloma cells, and supernatants screened on COS cells expressing ephrin-B2-GPI.

Supernatant from clone #6E3 bound well to ephrinB2 on live COS cells. The ephrin-B2-GPI COS cell lines are  
10 a pooled population of G418-selected cells, with 30% of the cells being positive for ephrinB2. Control (untransfected) COS cells were negative when stained with the same antibody.

Example 13: Some anti-ephrin-B2 antibodies block the  
15 EphB2-ephrinB2 binding interaction

In cases where the antibodies also block function (i.e., inhibit binding of ephrin-B2 to its receptors), they can be used as potential anti-angiogenic agents. To demonstrate an assay to identify such  
20 function-inhibiting antibodies, we screened 12 hamster anti-EphrinB2 hybridoma supernatants for their ability to reduce the binding of GPI-ephrin-B2 (expressed on COS cells) to a soluble EphB2-Fc fusion protein. Binding was detected by <sup>125</sup>I-labeled goat anti-human Fc antibody.  
25 Pre-incubation of cells with various supernatants revealed that the majority of the antibodies have no blocking effects on subsequent receptor-ligand binding (control as 100%), even though these supernatants all contained antibodies that bound to ephrin-B2-GPI. One  
30 of the antibodies produced a 40% reduction in EphB2-Fc binding to the ephrin-B2-GPI COS cells (Figure 2).

## EQUIVALENTS

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims. Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the claims.

## CLAIMS

What is claimed is:

1. A method for altering angiogenesis in a mammal,  
comprising administering to the mammal, in a  
therapeutically effective quantity, a drug which  
alters binding or interaction of an artery-specific  
cell surface molecule with a vein-specific cell  
surface molecule.
2. The method of Claim 1 wherein the artery-specific  
cell surface molecule is an artery-specific ligand  
or receptor and the vein-specific cell surface  
molecule is a vein-specific receptor or ligand.
3. The method of Claim 1 wherein the artery-specific  
cell surface molecule is an Ephrin family ligand  
and the vein-specific cell surface molecule is an  
Eph family receptor.
4. The method of Claim 3 wherein angiogenesis is  
inhibited and the drug interferes with binding or  
interaction of the artery-specific Ephrin family  
ligand with the vein-specific Eph family receptor.
5. The method of Claim 3 wherein angiogenesis is  
enhanced and the drug enhances binding or  
interaction of the artery-specific Ephrin family  
ligand with its vein-specific Eph family receptor.
6. The method of Claim 3 wherein the drug is an  
antagonist of the artery-specific Ephrin family  
ligand or an antagonist of the vein-specific Eph  
family receptor.

7. The method of Claim 3 wherein the artery-specific Ephrin family ligand is EphrinB2 and the vein-specific Eph family receptor is EphB4.
- 5 8. A method for selectively delivering a drug to arteries in a mammal, comprising administering to the mammal a complex comprising:
  - (a) the drug and
  - (b) a component which binds an artery-specific cell surface molecule,
- 10 under conditions appropriate for the component of (b) to bind the artery-specific cell surface molecule, whereby the drug is delivered to arteries.
9. The method of Claim 8, wherein the artery-specific cell surface molecule is a ligand or receptor.
- 15 10. The method of Claim 8, wherein the artery-specific cell surface molecule is an Ephrin family ligand.
11. The method of Claim 10 wherein the drug is an anti-angiogenic drug and the component of (b) is an antibody specific for the artery-specific Ephrin family ligand, or a receptor of the artery-specific Ephrin family ligand.
- 20 12. The method of Claim 10 wherein the artery-specific Ephrin family ligand is EphrinB2.
- 25 13. The method of Claim 12 wherein the drug is an anti-angiogenic drug.
14. The method of Claim 12 wherein the drug is an angiogenic drug.

15. The method of Claim 12 wherein the drug inhibits formation of atherosclerotic plaques.
16. A method for selectively delivering a drug to veins in a mammal, comprising administering to the mammal  
5 a complex comprising:
  - (a) the drug, and
  - (b) a component which binds a vein-specific cell surface molecule,  
under conditions appropriate for the component of  
10 (b) to bind the vein-specific cell surface molecule, whereby the drug is delivered to veins.
17. The method of Claim 16 wherein the vein-specific cell surface molecule is a receptor or ligand.
18. The method of Claim 16, wherein the vein-specific  
15 cell surface molecule is a vein-specific Eph family receptor.
19. The method of Claim 18 wherein the drug is an anti-angiogenic drug and the component of (b) is an antibody specific for the vein-specific Eph family  
20 receptor, or a ligand of the vein-specific Eph family receptor.
20. The method of Claim 18 wherein the vein-specific Eph family receptor is EphB4.
21. The method of Claim 20 wherein the drug is an anti-  
25 angiogenic drug.
22. The method of Claim 20 wherein the drug is an angiogenic drug.

23. A transgenic mouse having an indicator gene which is detectably expressed in cells of arteries but not cells of veins.
- 5 24. The mouse of Claim 23 wherein the indicator gene is inserted in an artery-specific Ephrin family ligand gene.
25. A transgenic mouse of genotype *EphrinB2*<sup>+/-</sup>.
26. A transgenic mouse in which *EphrinB2* genes comprise an insertion that marks all arteries but not veins.
- 10 27. A transgenic mouse of genotype *EphrinB2*<sup>taulacz/+</sup>.
28. A method for identifying artery cells in a mouse having an indicator gene inserted in one or more alleles of *EphrinB2*, comprising staining a section of the mouse with a substance appropriate for  
15 detection of expression of the indicator gene.
29. A transgenic mouse having an indicator gene which is expressed in venous endothelial cells but not in arterial endothelial cells.
30. The mouse of Claim 29 wherein the indicator gene is  
20 inserted in a vein-specific Eph family receptor gene.
31. The transgenic mouse of Claim 30 wherein the vein-specific Eph family receptor gene encodes EphB4.
32. A transgenic mouse of genotype *EphB4*<sup>+/-</sup>.



33. A method for identifying vein cells in a mouse having an indicator gene inserted in one or more alleles of *EphB4*, comprising staining a section of the mouse with a substance appropriate for  
5 detection of expression of the indicator gene.
34. A method for testing an effect of a drug on growth of arteries, comprising administering the drug to a mouse having an indicator gene inserted in a gene specifically expressed in arteries, observing the  
10 effect of the drug, and comparing the effect to that produced in a suitable control mouse.
35. A method for testing an effect of a drug on growth of veins, comprising administering the drug to a mouse having an indicator gene inserted in a gene specifically expressed in veins, observing the  
15 effect of the drug, and comparing the effect to that produced in a suitable control mouse.
36. A method for identifying an arterial cell in a tissue sample from a mammal, comprising contacting  
20 the tissue sample with a molecule which binds to EphrinB2, wherein said molecule is linked to a label, and detecting the label, wherein if label is detected on a cell, the cell is an arterial cell.
37. The method of Claim 36, wherein said molecule is an  
25 antibody.
38. A method for identifying venous endothelial cells in a tissue sample, comprising contacting the tissue sample with a molecule which binds to *EphB4*, wherein said molecule is linked to a label, and

detecting the label, wherein if label is detected on a cell, the cell is a venous endothelial cell.

39. The method of Claim 38, wherein said molecule is an antibody.
- 5 40. A method for directing a substance to arteries in a mammal, comprising administering to the mammal a complex which comprises the substance linked to a moiety which binds EphrinB2.
- 10 41. A method for directing a substance to veins in a mammal, comprising administering to the mammal a complex which comprises the substance linked to a moiety which binds EphB4.
- 15 42. A method for altering development of blood vessels in a mammal, comprising administering to the mammal a soluble polypeptide comprising the extracellular domain of an artery-specific cell surface protein or a soluble polypeptide comprising the extracellular domain of a vein-specific cell surface protein.
- 20 43. The method of Claim 42 wherein the artery-specific cell surface protein is an Ephrin family ligand and the vein-specific cell surface protein is an Eph family receptor.
- 25 44. The method of Claim 43 wherein the Ephrin family ligand is EphrinB2 and the Eph family receptor is EphB4.

45. A method for identifying a drug that inhibits interaction of an arterial cell-specific surface molecule with a venous cell-specific surface molecule, comprising:
- 5 (a) combining:
- (1) the arterial cell-specific surface molecule;
- (2) the venous cell-specific surface molecule; and
- 10 (3) a drug to be assessed for its ability to inhibit interaction between the molecule of (1) and the molecule of (2), under conditions appropriate for interaction between the molecule of (1) and the
- 15 molecule of (2);
- (b) determining the extent to which the molecule of (1) and the molecule of (2) interact; and
- (c) comparing the extent determined in (b) with
- 20 the extent to which interaction of the molecule of (1) and the molecule of (2) occurs in the absence of the drug to be assessed and under the same conditions appropriate for interaction of the molecule of (1) with the molecule of (2);
- 25 wherein if the extent to which interaction of the molecule of (1) and the molecule of (2) is less in the presence of the drug than in the absence of the drug, the drug is one which inhibits interaction of the arterial cell-specific molecule of (1) with the
- 30 venous cell-specific molecule of (2).
46. The method of Claim 45 wherein the arterial cell-specific surface molecule is an Ephrin family

ligand and the venous cell-specific surface molecule is an Eph family receptor.

47. The method of Claim 46 wherein the Ephrin family ligand is EphrinB2 and the Eph family receptor is EphB4.
48. A method for identifying a drug that enhances interaction of an arterial cell-specific surface molecule with a venous cell-specific surface molecule, comprising:
- (a) combining:
    - (1) the arterial cell-specific surface molecule;
    - (2) the venous cell-specific surface molecule; and
    - (3) a drug to be assessed for its ability to inhibit interaction between the molecule of (1) and the molecule of (2), under conditions appropriate for interaction between the molecule of (1) and the molecule of (2);
  - (b) determining the extent to which the molecule of (1) and the molecule of (2) interact; and
  - (c) comparing the extent determined in (b) with the extent to which interaction of the molecule of (1) and the molecule of (2) occurs in the absence of the drug to be assessed and under the same conditions appropriate for interaction of the molecule of (1) with the molecule of (2);
- wherein if the extent to which interaction of the molecule of (1) and the molecule of (2) is greater in the presence of the drug than in the absence of

the drug, the drug is one which enhances interaction of the arterial cell-specific molecule of (1) with the venous cell-specific molecule of (2).

- 5 49. The method of Claim 48 wherein the arterial cell-specific surface molecule is an Ephrin family ligand and the venous cell-specific surface molecule is an Eph family receptor.
- 10 50. The method of Claim 49 wherein the Ephrin family ligand is EphrinB2 and the Eph family receptor is EphB4.
- 15 51. A method for isolating arterial endothelial cells, comprising dissociating cells of a tissue sample comprising arterial endothelial cells, contacting the dissociated cells with a substance which binds to a cell-surface protein expressed specifically on arterial endothelial cells, and separating the cells which have bound the substance from cells which have not bound the substance.
- 20 52. A method for isolating arterial endothelial cells, comprising dissociating cells of a tissue sample comprising arterial endothelial cells, contacting the cells with a substance which binds to a cell-surface protein expressed specifically on arterial endothelial cells, wherein said substance is bound to a solid support, removing cells which do not bind to the substance, and releasing the arterial endothelial cells from the solid support.
- 25

53. A method for isolating venous endothelial cells, comprising dissociating cells of a tissue sample comprising venous endothelial cells, contacting the dissociated cells with a substance which binds to a cell-surface protein expressed specifically on venous endothelial cells, and separating the cells which have bound the substance from cells which have not bound the substance.
54. A method for isolating venous endothelial cells, comprising dissociating cells of a tissue sample comprising venous endothelial cells, contacting the cells of the tissue sample with a substance which binds to a cell-surface protein expressed specifically on venous endothelial cells, wherein said substance is bound to a solid support, removing cells which do not bind to the substance, and releasing the venous endothelial cells from the solid support.
55. Isolated arterial endothelial cells.
56. Isolated venous endothelial cells.
57. A method for assessing an effect of one or more drugs on arteries, comprising adding one or more drugs to isolated arterial cells, and observing the cells for the effect.
58. The method of Claim 57 wherein the cells are endothelial cells.
59. A method for assessing an effect of one or more drugs on veins, comprising adding one or more drugs

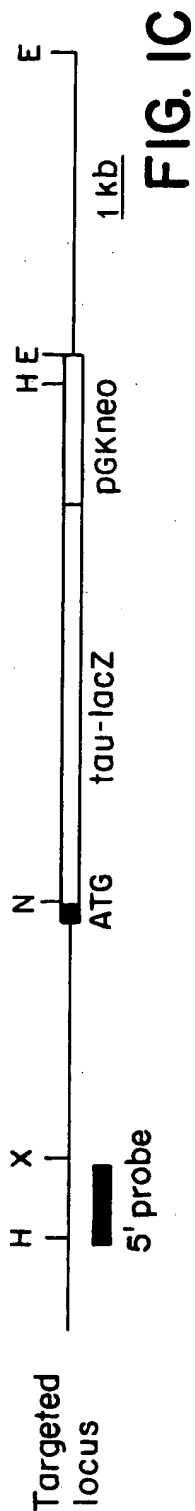
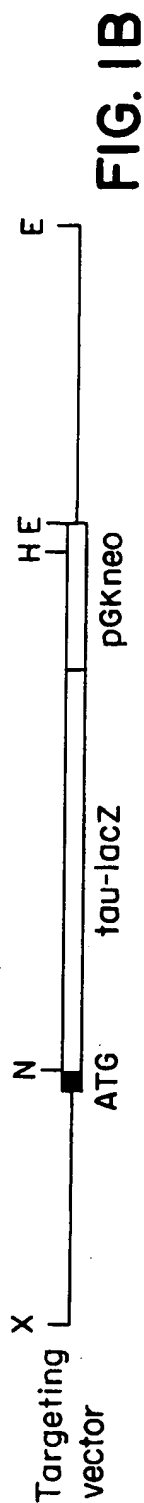
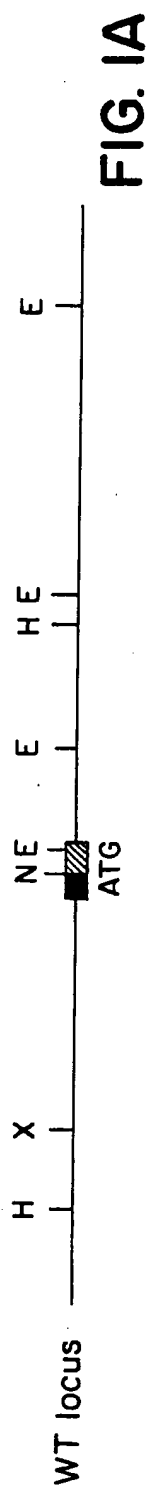
to isolated venous endothelial cells, and observing the cells for the effect.

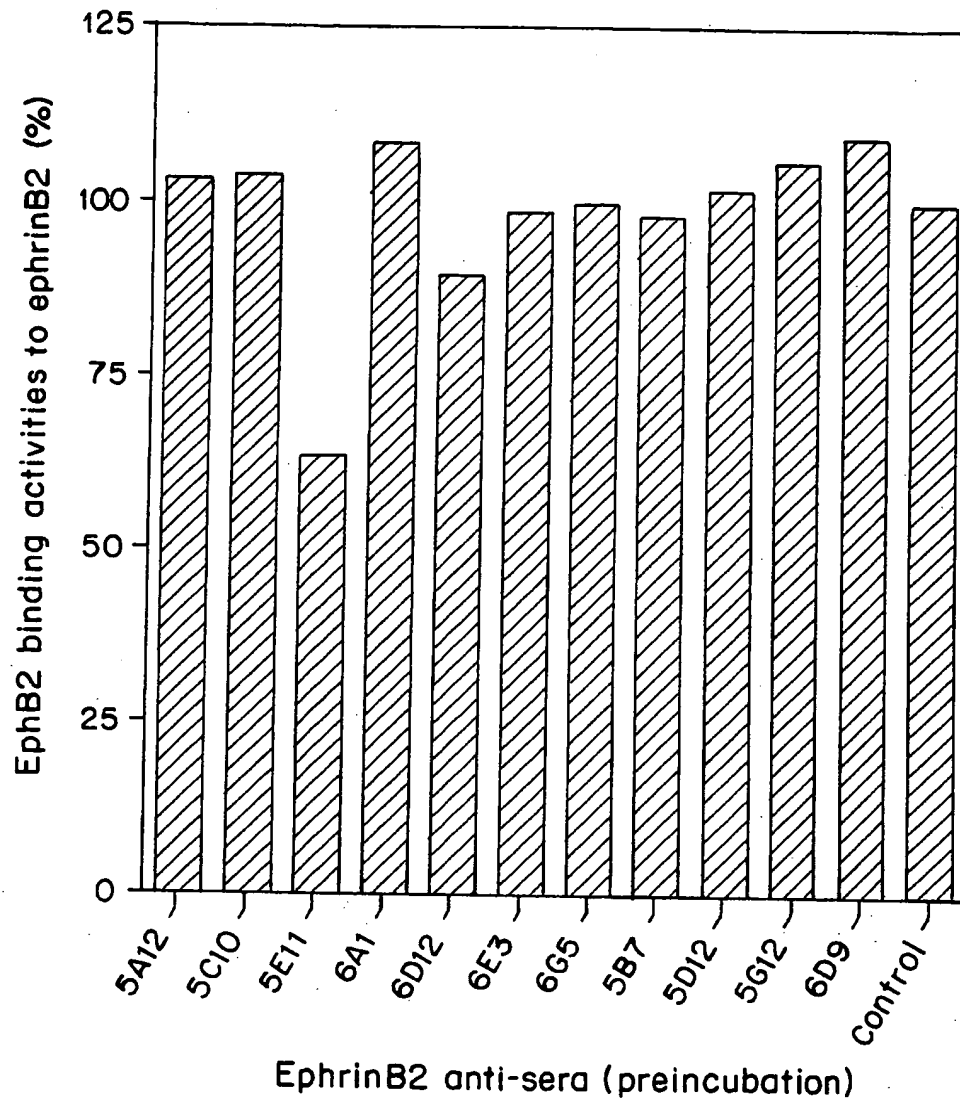
60. A cell line derived from arterial endothelial cells.
- 5 61. A cell line derived from venous endothelial cells.
62. A cell line which produces a protein that is detectably produced in arteries, but is not detectably produced in veins.
63. A cell line which produces a protein that is  
10 detectably produced in veins, but is not detectably produced in arteries.
64. A cDNA library produced from isolated arterial endothelial cells.
65. A cDNA library produced from isolated venous  
15 endothelial cells.
66. A method for identifying a gene which shows differential expression in venous endothelial cells compared to arterial endothelial cells, comprising producing a transgenic mouse having an indicator  
20 insertion gene in a gene to be tested for differential expression, and observing expression of the indicator insertion gene, wherein a difference in expression of the indicator insertion gene in venous endothelial cells and arterial  
25 endothelial cells indicates a gene which shows differential expression.

67. A method for modifying arteries in a mammal, comprising genetically altering isolated arterial endothelial cells and introducing the altered cells into the mammal.
- 5 68. A method for modifying veins in a mammal, comprising genetically altering isolated vein endothelial cells and introducing the altered cells into the mammal.
- 10 69. A method for altering angiogenesis in a tumor of a mammal, comprising administering to the mammal, in a therapeutically effective quantity, a drug which alters binding or interaction of an artery-specific cell surface molecule with a vein-specific cell surface molecule.
- 15 70. The method of Claim 69 wherein angiogenesis is inhibited and the drug interferes with binding or interaction of the artery-specific cell surface molecule with the vein-specific cell surface molecule.
- 20 71. The method of Claim 70 wherein the artery-specific cell surface molecule is an Ephrin family ligand and the vein-specific cell surface molecule is an Eph family receptor.
- 25 72. The method of Claim 71 wherein the Ephrin family ligand is EphrinB2 and the Eph family receptor is EphB4.



73. A method for inhibiting angiogenesis in a mammal, comprising administering to the mammal an agent that binds to EphrinB2.
74. A method for identifying a clone comprising an artery-specific gene, comprising:
- a) isolating arterial endothelial cells;
  - b) producing cDNA from the isolated cells;
  - c) producing a cDNA library from the cDNA;
  - d) screening the library, by DNA hybridization, with cDNA library probes produced from isolated arterial endothelial cells, and with cDNA library probes produced from isolated venous endothelial cells; and
  - e) identifying the clone comprising an artery-specific gene by its differential hybridization to probes produced from isolated arterial endothelial cells.
75. An antibody that binds to the extracellular domain of EphrinB2.
76. An antibody that binds to the extracellular domain of EphB4.



**FIG. 2**

(19) World Intellectual Property Organization  
International Bureau



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21 October 1999 (21.10.1999)

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(10) International Publication Number  
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(75) Inventors/Applicants (for US only): WANG, Hai, U. [CN/US]; Room 217, 1-86 Heck House, Pasadena, CA 91125 (US). CHEN, Zhouleng [CN/US]; 1317 North Holliston Avenue, No. 4, Pasadena, CA 91125 (US). ANDERSON, David, J. [US/US]; 2108 Glenview Terrace, Altadena, CA 91001 (US).
- (74) Agents: BROOK, David, E. et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02421-4799 (US).
- (81) Designated States (national): AU, CA, JP, US.
- (84) Designated States (regional): European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).
- (63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:  
US 09/085,820 (CIP)  
Filed on 28 May 1998 (28.05.1998)
- Published:  
— With international search report.
- (88) Date of publication of the international search report:  
12 July 2001
- (71) Applicant (for all designated States except US): CALIFORNIA INSTITUTE OF TECHNOLOGY [US/US]; 1200 East California Boulevard, Pasadena, CA 91125 (US).
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 99/52541 A3

(54) Title: ARTERY- AND VEIN-SPECIFIC PROTEINS AND USES THEREFOR

(57) Abstract: Arterial and venous endothelial cells are molecularly distinct from the earliest stages of angiogenesis. This distinction is revealed by expression on arterial cells of a transmembrane ligand, called EphrinB2 whose receptor EphB4 is expressed on venous cells. Targeted disruption of the *EphrinB2* gene prevents the remodeling of veins from a capillary plexus into properly branched structures. Moreover, it also disrupts the remodeling of arteries, suggesting that reciprocal interactions between pre-specified arterial and venous endothelial cells are necessary for angiogenesis. This distinction can be used to advantage in methods to alter angiogenesis, methods to assess the effect of drugs on artery cells and vein cells, and methods to identify and isolate artery cells and vein cells, for example.

# INTERNATIONAL SEARCH REPORT

International Application No

PCI/US 99/08098

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K38/17 A61K38/19 A01K67/027 C12N5/06 C12N5/08  
G01N33/50 C12Q1/68 A61K35/12 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 26958 A (HARVARD COLLEGE) 6 September 1996 (1996-09-06) page 4, line 7-14 page 28, line 19-33 claims 14-22,27	25, 62, 75
X	BENNETT B D ET AL: "Molecular cloning of a ligand for the EPH-related receptor protein -tyrosine kinase Htk" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 92, March 1995 (1995-03), pages 1866-1870, XP002010465 ISSN: 0027-8424 abstract page 1866, right-hand column, line 26 page 1869, left-hand column, line 18,19 -/-	63,76

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents :

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"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search

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Date of mailing of the international search report

11/11/1999

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# INTERNATIONAL SEARCH REPORT

International Application No

PL 1/US 99/08098

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SIMONET, SERGE ET AL: "Venous and arterial endothelial cells respond differently to thrombin and its endogenous receptor agonist" EUR. J. PHARMACOL. (1992), 216(1), 135-7 , XP002120943 abstract page 136, right-hand column, line 41-43 ---	8,9,16, 17
A	EP 0 633 315 A (INVEST ENERGET MEDIOAMBIENT) 11 January 1995 (1995-01-11) claims ---	23-32
P,X	WANG H U ET AL: "Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin - B2 and its receptor Eph - B4 'see comments!.' CELL, (1998 MAY 29) 93 (5) 741-53. , XP002120944 the whole document ---	1-76
P,X	YANCOPOULOS, G.D. ET AL: "Vasculogenesis, Angiogenesis, and Growth Factors: Ephrins Enter the Fray at the Border" CELL, (19980529) VOL. 93, NO. 5, PP. 661-664. ISSN: 0092-8674., XP002120945 Children's Hospital and Harvard Medical School, Boston, MA 02115, USA figure 1 ---	1-22, 33-76
P,X	CHEMICAL ABSTRACTS, vol. 130, no. 24, 14 June 1999 (1999-06-14) Columbus, Ohio, US; abstract no. 323337, WANG, HAI: "Transmembrane ephrin ligands in neural and vascular development" XP002120946 abstract & (1998) 116 PP. AVAIL.: UMI, ORDER NO. DA9912892 FROM: DISS. ABSTR. INT., B 1999, 59(11), 5721, -----	1-76

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/08098

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 1-22,40-44,67-73 are directed to a method of treatment of the human/animal body, and the claims 27,33,45-50,66 (partially) 34,35 (complete) are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

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Continuation of Box I.1

Claims Nos.: 1-22 40-44 67-73 27 33 45-50 66 (partially) 34 35  
(complete)

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

Rule 39.1(iv) PCT - Diagnostic method practised on the human or animal body Rule 39.1(iv)



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/08098

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9626958	A	06-09-1996	NONE	
EP 0633315	A	11-01-1995	JP 8019352 A	23-01-1996